Active-Site Mutagenesis of *E. coli* Alkaline Phosphatase: Replacement of Serine-102 with Nonnucleophilic Amino Acids[1]

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The active-site nucleophile in bacterial alkaline phosphatase, Ser-102, was replaced using site-directed mutagenesis, with leucine and, separately, alanine. Remarkably, the mutant enzymes S102A and S102L, which lack a nucleophilic side chain at position 102, still catalyze the hydrolysis of phosphate monoesters. The k_{cat} values are, respectively, ~1/1000 and ~1/500 of the wild-type enzyme. The mutant enzymes have K_m values, K_i values (inorganic phosphate), and pH profiles that are similar to wild-type. Several experiments argue against the possibility of wild-type contamination. The S102L mutant has a substrate-dependent partition ratio which is consistent with a change in the enzyme mechanism. Direct hydrolysis by zinc-activated water may account for the catalytic activity of these mutant enzymes. This work illustrates the use of site-directed mutagenesis to uncover latent enzyme activity and reinforces the idea that the zinc atoms in the active site are the critical structural feature in the design of alkaline phosphatase.

Bacterial alkaline phosphatase (BAP; E.C. 3.1.3.1) is a metalloenzyme that catalyzes the nonspecific hydrolysis of phosphate monoesters.[2] The mature enzyme is a dimer of molecular weight 94 058, and the complete amino acid sequence has been determined.[3] The catalytic mechanism has been the subject of numerous kinetic[4] and structural studies.[5] The generally accepted catalytic mechanism involves four steps including the transient formation of a covalent phosphoryl-enzyme intermediate (E-P_i) with Ser-102 (eq 1). Subsequently, water (hydrolysis) or an

$$E + ROP_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^*ROP_i \underset{k_{-2}[ROH]}{\overset{k_2}{\longrightarrow}} E\text{-}P_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E^*P_i \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P_i \quad (1)$$

alternative nucleophilic acceptor such as an alcohol (transphosphorylation), dephosphorylates the phosphoryl-enzyme intermediate. The wild-type enzyme exhibits maximal activity around pH 8 where the rate-limiting step is release of the tightly bound inorganic phosphate (k_4), P_i, from the enzyme product complex. Accordingly, the rate of the enzyme-catalyzed reaction is essentially independent of the pK_a of the substrate leaving group and inorganic phosphate is a competitive inhibitor.[4] At pH <5.5 the phosphoserinyl intermediate is stable (k_3 is rate limiting) and has been observed by ^{31}P NMR.[6] Stereochemical analysis of the enzyme-catalyzed transphosphorylation with chiral *p*-nitrophenyl (R_p)-[^{16}O,^{17}O,^{18}O]phosphate has shown that the reaction proceeds with overall retention of configuration at phosphorus.[7] This result has been ascribed to a double in-line nucleophilic displacement by Ser-102 and water.

The X-ray crystal structure of BAP has recently been reported to 2.0-Å resolution in the presence of inorganic phosphate (E*P_i).[8] The active site is a small pocket containing a tight cluster of 2 zinc ions (3.9-Å separation) and one magnesium ion (5 and 7 Å from the two zinc atoms). Inorganic phosphate completely fills the pocket. There is no apparent binding pocket for the R group of the phosphate monoester consistent with the nonspecificity of the enzyme. The zinc atoms are well positioned to activate Ser-102 and water for the two nucleophilic attacks required in the enzyme mechanism.

Site-directed mutagenesis has been used to explore the role of specific amino acid residues in the active site of BAP. Replacement of Ser-102 with Cys produced a mutant which had ~25% of the activity (k_{cat}, PNPP, 50 mM Tris, 50 mM NaCl, pH 7.5) of the wild-type enzyme.[9] In contrast to the wild-type, the reaction optimum shifted to slightly lower pH, and V_{max} was found to be dependent upon the pK_a of the substrate's leaving group. Stereochemical studies with the S102C mutant indicated that the enzyme catalyzed the transphosphorylation reaction with overall retention of configuration at phosphorus.[10] Butler-Ransohoff concluded from these studies that the S102C mutation conserved the overall mechanism but changed the rate determining step from release of inorganic phosphate from E*P_i to phosphorylation (k_2) of the active-site thiol group at 102. The S102C mutation in BAP has consequences that are similar to the Glu to Asp mutation in triose phosphate isomerase.[11] In both cases the overall chemical mechanism is retained but the mutation changes the relative free energy of the transition states of the individual steps and results in a different rate-limiting step.

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(1) Abbreviations: BAP, bacterial alkaline phosphatase; Tris, tris(hydroxymethyl)aminomethane; P_i, inorganic phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PNPP, 4-nitrophenyl phosphate; β-Naphth, β-naphthyl phosphate; MUP, methylumbelliferyl phosphate; DNPP, 2,4-dinitrophenyl phosphate.

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Table I. Michaelis-Menten Parameters of the Mutant Alkaline Phosphatases S102L and S102A (1 M Tris, pH 8.0, 25 °C)

enzyme	substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
none	PNPP	$(5.5 \pm 0.2) \times 10^{-9}$		
wild-type	PNPP	31.0 ± 0.05	19.5 ± 0.2	1.6×10^6
S102L	PNPP	0.070 ± 0.001	17.6 ± 1.0	4.0×10^3
S102L	β-Naphth	0.064 ± 0.004	15.8 ± 0.5	4.1×10^3
S102L	DNPP	0.075 ± 0.003	17.9 ± 0.3	4.2×10^3
S102A	PNPP	0.036 ± 0.007	27.6 ± 2.8	1.3×10^3

Arg-166 is located near the entrance to the active site and has been the focus of several mechanistic studies. A Bronsted plot of log (k_{cat}/K_m) vs pK_a of the substrate's leaving group demonstrated that the change in effective charge on the leaving group is small.[12] It was proposed that strong electrophilic participation by either Arg-166, which is located near the entrance to the active site, or by a zinc atom was involved in the charge neutralization. Previous studies have shown that covalent modification of Arg-166 with phenyl glyoxal or 2,3-butanedione completely inactivated the enzyme and led investigators to believe that this residue was intimately involved in the catalytic process.[13] Mutagenesis experiments greatly clarified the role of Arg-166. Replacement of Arg-166 with Lys or Glu[14] and Ser or Ala[15] produced competent mutant enzymes with k_{cat}/K_m values varying from 24% to 1% of the wild-type enzyme. These studies indicate that Arg-166 plays a role in the binding of substrate and inorganic phosphate. However, Arg-166 is not essential for activity, and electrophilic interaction at 166 does not significantly contribute to the charge neutralization of the alkoxide leaving group. Consequently, it was proposed that one of the active-site zinc atoms is responsible for the stabilization of the leaving group.

We have continued to probe the structural and functional requirements that are necessary for catalysis by substituting the nonnucleophilic amino acids Ala and Leu for Ser-102. Despite the lack of a protein-based nucleophile in the active site, we report weak phosphatase activity in these mutants.[16]

Results and Discussion

Steady-State Kinetic Studies. Both mutant enzymes Ala-102 (S102A) and Leu-102 (S102L) catalyze the hydrolysis of phosphate monoesters with greatly reduced facility. Table I compares the values of k_{cat} and K_m for the mutant enzymes and the wild-type enzyme. With *p*-nitrophenyl phosphate (PNPP) as a substrate the k_{cat}/K_m for the S102L mutant is reduced by a factor of 400 and the k_{cat}/K_m for the S102A mutant is reduced by a factor of ~1000. The differences are almost entirely due to the low k_{cat} values. Although the mutant enzymes' activity is greatly reduced relative to wild-type, the hydrolysis of PNPP is enhanced by a factor of $\sim 10^7$ compared to the uncatalyzed reaction. An interesting feature illustrated in Table I is the apparent insensitivity of the k_{cat} values for S102L with substrates of widely different pK_a's. A similar trend has also been observed with S102A mutant where the relative V_{max} values are: PNPP, 100%, β-naphthyl phosphate (β-Naphth), 73%; methylumbelliferyl phosphate (MUP), 87%. The lack of dependence of k_{cat} with substrate has been thoroughly documented for the wild-type enzyme.[12] The stability of the leaving group does not significantly contribute to the rate at which the wild-type or mutant enzymes turn over.

Table II. Inhibition of Wild-Type and Mutant Enzymes by Inorganic Phosphate (1 M Tris, pH 8.0, 25 °C)

enzyme	K_i (μM)
wild-type	5.6 ± 0.7
S102L	5.8 ± 0.4
S102A	7.9 ± 0.3

Table III. Transphosphorylation Ratio: [ROH]/[P_i] (1 M Tris, pH 8.0, 25 °C)

enzyme	PNPP	MUP	β-Napth
wild-type	2.57 ± 0.05	2.59 ± 0.12	2.31 ± 0.05
S102L	1.84 ± 0.09	2.75 ± 0.25	3.71 ± 0.24

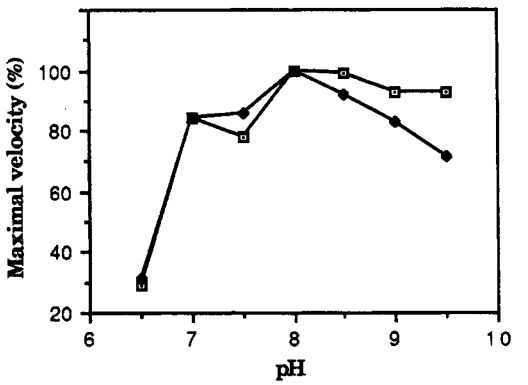

Figure 1. pH dependence of the maximal velocities of wild-type BAP (□) and S102L (◆). Reaction conditions: 1.0 M Tris, 5 mM PNPP, 1.0 M IS (NaCl), 25 °C.

Inorganic phosphate is a competitive inhibitor of BAP and the mutant enzymes. The K_i values (Table II) for these enzymes are rather similar, indicating that electrostatic interactions of the anionic phosphate with the positive metal centers and the cationic Arg-166 are conserved in the active site of the mutant enzymes.

Like BAP, the k_{cat} values for S102L is enhanced by increasing Tris concentrations. When the Tris concentration was varied from 0.01 to 1.0 M, the k_{cat} increased by ~25% compared to an 80% increase for wild-type. This increase is due solely to an increase in the rate of Tris phosphorylation (transphosphorylation) and not to an increase in inorganic phosphate formation (hydrolysis).

It has long been established that the partition ratio of the wild-type enzyme between water (hydrolysis) and Tris (transphosphorylation) gives a constant product ratio that is independent of the substrate.[17] These data provided strong evidence that the hydrolysis of a wide variety of substrates proceeded through a common phosphoenzyme intermediate. In contrast to the behavior of the wild-type enzyme, the S102L mutant shows a substrate-dependent partition ratio (Table III). This result is consistent with the replacement of the nucleophilic serine residue with the nonnucleophilic aliphatic side chain of leucine, which cannot form a common covalent intermediate. These results require that the partitioning between hydrolysis and transphosphorylation must occur on the enzyme-bound phosphate monoester, or shortly after the phosphate ester linkage has been broken while the leaving group is still in the vicinity of the active pocket.

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Figure 1 displays the relative maximal reaction velocities for the S102L enzyme at V_{max} as a function of pH. The S102L mutant shows a slight narrowing of the optimum range at high Tris concentration, but the overall pH profiles of the mutant and the wild-type enzymes remains similar. These data suggest that the activity of the enzymes are controlled by the ionization of similar if not identical functionalities in the active site. Previously it has been suggested that the pH profile of the WT may be controlled by the ionization of water-on-zinc, which dephosphorylates the serinyl phosphate intermediate.[4] A similar motif may be operating in the mutants lacking the nucleophilic serine residue where a zinc-activated water directly attacks the enzyme substrate complex, E*ROP$_i$.

Integrity of Mutant Proteins. The mutant enzymes S102A and S102L have many similarities in their steady-state kinetic behavior with the wild-type enzyme: K_m, pH profiles, and V_{max} independent of substrate. These data led us to consider the possibility that our mutant enzyme preparations were contaminated with a low level of the wild-type enzyme.[18]

The *E. coli* bacterial host AW1043 has a deletion in its *pho*A gene which includes the region coding for the active site of the enzyme. Control experiments in which AW1043 was grown without the pBR322-BAP plasmid (untransformed cells) showed no significant phosphatase activity following the standard isolation and purification protocol. Under the conditions employed we estimate that we would have been able to detect as low as ~25% of the activity observed for the S102L mutant enzyme.

In a similar mutagenesis study with β-lactamase (a nonmetalloenzyme) it was found that replacement of the active-site nucleophilic Ser-68 residue with Gly (AGC to GGC) produced an enzyme with 0.001 the k_{cat} of the wild-type enzyme.[19] However, when the point mutation was constructed with a different codon specifying the same substitution (AGC to GGA), the isolated enzyme had no detectable activity. This behavior was attributed to a codon specific low frequency misreading of the m-RNA during translation to produce small amounts of the wild-type enzyme in the protein preparation. In order to address this possibility in the BAP system, the S102L mutation was constructed with a second codon sequence (first construction, TCG to CTT; and second construction, TCG to CTG). The second construction would require a double misreading of the codon to produce the wild-type sequence. The protein preparation produced by this plasmid gave similar Michaelis-Menten parameters to that found with the initial S102L mutant.

Expression of BAP is regulated with respect to the phosphate level of the extracellular medium. We considered the possibility that the low phosphate MOPS medium employed for cell growth was selecting against colonies harboring the mutant enzymes with low activity and selecting for active species that may result from low-frequency transcription errors and/or translation errors, reversions, or second-site mutations. If this were indeed the case, continuous growth under these stringent conditions should eventually give rise to a population enriched in the additional mutation. To test this possibility, the bacterial colonies transformed with the S102L mutant plasmid were grown on low phosphate (0.1 mM KH_2PO_4) MOPS agar plates containing 5-bromo-4-chloro-3-indolyl phosphate as an indicator for alkaline phosphatase activity. The S102L bacterial colonies were associated with a very faint blue color indicating weak alkaline phosphatase activity.[20] Individual colonies which displayed any deviation toward a more intense blue color were streaked on fresh MOPS low phosphate plates. This process was repeated for five rounds without any visual evidence of increased activity, and the colonies remained markedly different in color from those harboring the wild-type *pho*A gene. Furthermore, direct sequencing in the region around 102 of four isolets of the plasmid showed that the S102L mutation remained unaltered. Thus it does not appear that the mutant gene is undergoing low phosphate-induced selection pressure to revert to the wild-type or other phosphatase-active species.

As a final test for wild-type impurities we hoped to titrate the mutant enzyme preparations with a serine-specific active-site inhibitor. However despite a report in the literature,[21] we were unable to deactivate the wild-type enzyme upon prolonged incubation with diisopropyl fluorophosphate.[22] We are not aware of any other serine-specific inhibitors of BAP.

Mechanistic Consequences. It appears that the low-level phosphatase activity observed is attributable to the mutant enzymes S102A and S102L which lack a nucleophilic residue at position 102. Examination of the crystal structure of BAP at 2.8-Å resolution[5] does not reveal an alternative enzyme-based nucleophile that could participate in the "normal" phosphorylation–dephosphorylation mechanism. In the wild-type enzyme, Ser-102 is activated by one (or both) of the active-site zincs, and removal of this residue may open a ligand site for water. In fact the crystal structure of the wild-type suggests that in the absence of substrate, a water is coordinated to one of the zincs.[5] A zinc-activated water would be well positioned for a direct nucleophilic attack on the phosphate ester and may account for the activity of the mutant enzymes. The substrate-dependent partition ratio (Table III) for the competition between hydrolysis and transphosphorylation is consistent with a mechanism involving a single nucleophilic displacement. However, as we have previously demonstrated in the case of replacement of Ser-102 with Cys (S102C),[10] the variability in partition ratios is a necessary but not sufficient condition for establishing an alternative catalytic pathway. At present, the low activity of the mutant enzymes coupled with the low transphosphorylation ratio using butane-1,3-diol or propane-1,2-diol as acceptors have precluded analysis of the stereochemical course of transphosphorylation. The substrate-independent rates of hydrolysis suggest that release of inorganic phosphate from the enzyme–product complex may still be the rate-limiting step in enzyme turnover. Examination of the pre-steady-state kinetics should help clarify this issue.

Figure 2 shows the active-site region of the wild-type enzyme. The exact structure of the mutants containing the leucine and alanine substitutions at position 102 must await future crystallographic analysis. It would be expected that the large leucine side chain would necessarily cause some distortion in the active site in order to accommodate the steric bulk of the alkyl side chain. However, K_m values are similar to wild-type, and therefore processing is not fully perturbed.

This study illustrates the utility of site-directed mutagenesis to uncover latent enzymatic activity. That one can remove the active-site nucleophile, Ser-102 in BAP, and

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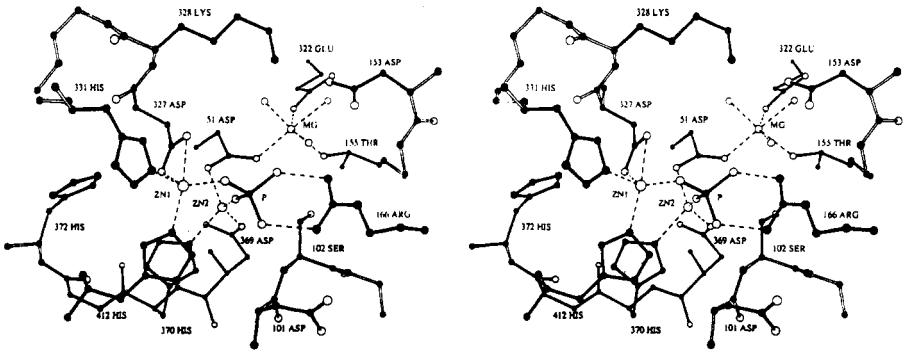

Figure 2. Stereodrawing of the active site of bacterial alkaline phosphatase at 2-Å resolution (water molecules omitted for clarity). Reprinted with permission from ref 8a. Copyright 1991 Academic Press Limited.

still produce a functional enzyme is in sharp contrast to the studies carried out on the nonmetalloenzyme β-lactamase.[19] Replacement of Ser-68 in β-lactamase with glycine abolished the activity of the enzyme. Although the exact mechanism employed by the BAP mutant enzymes S102L and S102A is not clear at this time, our results reinforce the notion that the zinc cluster in the active site is the key structural feature in the design of alkaline phosphatase.[14] The zinc atoms are critical in binding the substrate (and product), activating the nucleophilic attack(s), and stabilizing the alkoxide leaving group.

Experimental Section

Bacterial Strains and Media. *E. coli* strain AW1043 (Δ*lac gal*U *gal*K Δ(*leu-ara*) *pho*A-E15 *pro*C::Tn5) was used as the host cell for all experiments except site-directed mutagenesis, in which the male derivative AW1043F′ Tet [Δ*lac gal*U *gal*K Δ(*leu-ara*) *pho*A-E15 *pro*C::Tn5 F′Tet] was used.[23,24] Standard LB medium was used for general propagation of cells and for mutagenesis. For enzyme production and isolation, the host cells were grown in low phosphate (0.1 mM KH_2PO_4) containing MOPS medium[25] maintained at ~pH 7.8. All media contained ampicillin (250 mg/mL) and kanamycin (50 mg/mL).

Mutagenesis. The mutagenesis was performed using an M13 vector as previously described.[14] Specifically designed oligonucleotides (24-mers) were synthesized using the phosphoramidite method[30] to produce the desired mutations. The S102L mutation was carried out twice using two different oligonucleotides which contained degenerate codon sequences at the site of mutation (TCG → CTT for S102L, and TCG → CTG for S102L). The Ala mutation was effected with a single oligonucleotide (TCG → GCG for S102A). The double primer method of Zoller and Smith was used to construct the heteroduplex DNA.[26] Mutant plasmids were identified by colony screening with ^{32}P-labeled oligomer encoding the mutant sequence. The mutant gene was then sub-cloned into pBR322 between the *Bam*HI and *Hin*dIII sites. Direct DNA sequencing verified the presence of the mutation.

Production and Purification of the Mutant Enzymes. Transformed AW1043 cells were grown in 1 L of low phosphate MOPS media (with ampicillin and kanamycin, vide supra) at 37 °C until the o.d. at 600 nm reached ~2. The mutant enzymes were isolated from the cells following cold osmotic shock and ion-exchange chromatography as previously described.[14] The electrophoretic mobility of all mutants were identical to that of wild-type (SDS/polyacrylamide gel electrophoresis). Protein concentrations were measured from the absorbance at 278 nm ($\epsilon = 0.71$).[27] One liter of cell culture produced 1–3 mg of purified mutant enzyme.

Control Cell Growth. In order to verify that the plasmid-directed BAP synthesis was not being contaminated by phosphatase active proteins produced by the host cells, untransformed AW1043 cells were grown and subjected to the standard isolation and purification protocol. Analysis of seven ion-exchange chromatography fractions in which BAP consistently appeared in transformed cell growths showed no phosphatase activity in the untransformed cell growths (PNPP, 0.8M Tris, pH 8). The absorbance at 410 nm (ΔA_{410}) did not change more than 0.02 absorbance units after 30 min. Under comparable conditions the S102L mutant enzyme showed a ΔA_{410} of 0.1 absorbance units after 20 min. Examination of six additional fractions located around the usual BAP-containing fractions also showed no phosphatase activity. The total number of fractions in a typical ion-exchange chromatography run was 70 (4.5 mL per fraction).

Assays. The velocity of the enzyme-catalyzed reaction was followed spectrophotometrically: 4-nitrophenylate, $\epsilon_{410} = 1.6 \times 10^4$, pH 8.0, $pK_a = 7.16$;[28] β-naphtholate, $\epsilon_{325} = 770$, $pK_a = 9.24$.[12] Extinction coefficients were confirmed at different pH values after complete hydrolysis. All measurements were at 25 ± 0.1 °C in Tris·HCl buffer containing 10 mM $MgCl_2$ and 50 μM $ZnCl_2$. Values of k_{cat} and K_m were obtained from the Lineweaver–Burk plots of 6–10 points. Product inhibition was not considered in our analysis since only initial rates were used in the calculations. The partition ratio of transphosphorylation versus hydrolysis was determined by spectroscopically measuring the phenol concentration at pH 8 and the free phosphate concentration (Ames test).[29] The fraction of phosphate transferred to Tris was assumed to be the difference in the concentrations of phenol and free phosphate.

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